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Cloning and Expression of a *Streptococcus cremoris* Proteinase in *Bacillus subtilis* and *Streptococcus lactis*

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Previously, curing experiments suggested that plasmid pWV05 (17.5 megadaltons [Md]) of *Streptococcus cremoris* Wg2 specifies proteolytic activity. A restriction enzyme map of pWV05 was constructed, and the entire plasmid was subcloned in *Escherichia coli* with plasmids pBR329 and pACYC184. A 4.3-Md *Hin*dIII fragment could not be cloned in an uninterrupted way in *E. coli* but could be cloned in two parts. Both fragments showed homology with the 9-Md proteinase plasmid of *S. cremoris* HP. The 4.3-Md *Hin*dIII fragment was successfully cloned in *Bacillus subtilis* on plasmid pGKV2 (3.1 Md). Crossed immunoelectrophoresis of extracts of *B. subtilis* carrying the recombinant plasmid (pGKV500; 7.4 Md) showed that the fragment specifies two proteins of the proteolytic system of *S. cremoris* Wg2. PGKV500 was introduced in a proteinase-deficient *Streptococcus lactis* strain via protoplast transformation. Both proteins were also present in cell-free extracts of *S. lactis*(pGKV500). In *S. lactis*, pGKV500 enables the cells to grow normally in milk with rapid acid production, indicating that the 4.3-Md *Hin*dIII fragment of plasmid pWV05 specifies the proteolytic activity of *S. cremoris* Wg2.

In many lactic acid streptococci proteolytic activity is an unstable trait. Plasmid curing studies suggested that this activity is specified by plasmids in some strains (19, 20, 25, 26, 29). However, only in a few cases has it been established that this proteolytic ability was actually plasmid encoded by showing the cotransfer of plasmid and character (12, 15, 25). One characteristic of lactic streptococci which obscures the results of gene transfer and curing experiments is the multitude of different plasmids in these strains (8, 30). With plasmid-free strains, it was recently shown that polyethylene glycol-treated protoplasts of *Streptococcus lactis* take up free plasmid DNA (16, 17). Optimization of the protoplast transformation protocol enabled the direct cloning in *S. lactis* of a piece of DNA coding for the three phosphoenolpyruvate-dependent phosphotransferase system enzymes from a plasmid which was already known to specify lactose-fermenting ability in *S. lactis* (18). The recent development of a number of vectors, derived from the cryptic *S. cremoris* Wg2 plasmid pWV01, which replicate in *S. lactis*, *Bacillus subtilis*, and *Escherichia coli* has extended the application of molecular cloning techniques to the lactic streptococci (16). In the present communication we describe the cloning in *B. subtilis* and *S. lactis* of a part of the *S. cremoris* Wg2 plasmid pWV05, which encodes the proteolysis in this strain, in one of these pWV01-based vectors. Crossed immunoelectrophoresis (CIE) experiments with both organisms and growth studies with the transformed *S. lactis* strain indicate that the proteolytic activity of *S. cremoris* Wg2 is specified by a 4.3-megadalton (Md) *Hin*dIII fragment of pWV05.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used are described in Table 1. *B. subtilis* and *E. coli* cells were grown in TY broth (32). TY broth solidified with 1.5% agar was used for plating. Chloramphenicol and erythromycin were used at 5 μg/ml for *B. subtilis*; chloramphenicol, ampicilin, and tetracycline were used at 40, 30, and 12.5 μg/ml, respectively, for *E. coli*. Glucose-M17 broth and agar (34) were used to grow *S. lactis*. With *S. lactis*, chloramphenicol and erythromycin were used at 4 and 1 μg/ml, respectively. *S. cremoris* Wg2 and *S. cremoris* HP were routinely maintained in sterile 10% (wt/vol) reconstituted skim milk. Glycerophosphate-milk agar (23), slightly modified by the addition of 0.005% bromocresol purple, and citrate-milk agar (3) were used to test the ability of *S. lactis* strains to utilize milk protein.

Isolation of plasmid DNA. Plasmids from *E. coli* were isolated as described by Ish-Horowicz and Burke (15). Plasmid DNA from *B. subtilis* was isolated as described previously (16). This method also proved useful for minipreparations of plasmids from *S. lactis*. The method of LeBlanc and Lee (22) was used to isolate plasmids from *S. cremoris* Wg2 and *S. cremoris* HP.

Isolation of pWV05. Total plasmid DNA from *S. cremoris* Wg2 was separated in 0.5% agarose gels, using Tris-acetate buffer (40 mM Tris-acetate, 20 mM sodium acetate, 2 mM EDTA, pH 7.8). pWV05 was excised from the gels and processed for KI gradient centrifugation as described by Blin et al. (1) with the following modifications: gel slices were frozen (−20°C) and thawed, and approximately 12 g of solid KI was added to 6.5-g slices. After mixing on a blood cell suspension mixer to dissolve all agarose, 0.1 ml of ethidium bromide (5 mg/ml) was added, and the solution was adjusted to a refractive index of 1.444. The gradients were formed by centrifugation for 20 h at 53,000 rpm in a Beckman type 75Ti fixed-angle rotor at 20°C. A second run was needed to remove all agarose from the DNA band.

Restriction enzyme analysis and molecular cloning. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals and used according to the supplier's instructions. Digested DNA preparations were separated by electrophoresis in horizontal agarose gels (0.5 to 1%) in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, 0.5 μg of ethidium bromide per ml, pH 8.0). Restriction enzyme fragments of pWV05 were cloned in pBR329 or in pACYC184, with KI-purified pWV05. Alterna-

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant phenotype/genotype[a]	Remarks; plasmid mol wt ($\times 10^6$)	Source or reference
Bacillus subtilis PSL1	*arg leu thi* r^- m^- *stp recE4*		28
Escherichia coli C600	*thr leu thi lacY tonA phx supE vtr*		PC[b]
Streptococcus cremoris			
Wg2	Prt^+	17.5; 11.5; 6.1; 2.9; 1.5	29
Wg2	Prt^-	11.5; 2.9; 1.5	29
HP	Prt^+	25; 17.7; 9; 3.4; 1.9	10
HP	Prt^-	25; 17.7; 3.4; 1.9	10
Streptococcus lactis			
NCDO712	Lac^+ Prt^+	33; 9; 5.2; 2.5; 1.8	12
MG1363	Lac^- Prt^-	Plasmid-free derivative of *S. lactis* NCDO712	12
Plasmids			
pBR329	Cm^r Tc^r Ap^r		6
pACYC184	Cm^r Tc^r		4
pGD4	Cm^r	5.0-Md *Bam*HI fragment of pWV05 cloned in pACYC184	This work
pGD6	Cm^r Ap^r	pBR329 containing, among others, the 1.4-Md *Bam*HI-*Hin*dIII fragment of pWV05	This work
pGKV2	Cm^r Em^r	Double-resistance vector, constructed from the cryptic *S. cremoris* plasmid pWV01 (16)	J. M. B. M. van der Vossen et al. (manuscript in preparation)
pGKV500	Em^r	4.3-Md *Hin*dIII fragment of pWV05 cloned in pGKV2	This work

[a] Cm, Chloramphenicol; Em, erythromycin; Tc, tetracycline; Ap, ampicillin.
[b] PC, Phabagen Collection.

tively, specific fragments were purified from a digest of total *S. cremoris* Wg2 plasmid DNA by agarose gel electrophoresis and subsequent electroelution.

Competent cells of *E. coli* were transformed according to Mandel and Higa (24). Protoplasts of *B. subtilis* were transformed as described by Chang and Cohen (5). *S. lactis* protoplasts were prepared by using a modification of the method of Okamoto et al. (27). An overnight glucose-M17 culture was diluted 100-fold in glucose-M17 and incubated for 2 h at 30°C. The cells were washed in TMS (30 mM Tris-hydrochloride, 3 mM $MgCl_2$, 25% sucrose, pH 8.0) and resuspended to half of the original volume in TMS plus 30 μg of lysozyme per ml. After incubation for 1 h at 37°C, the protoplasts were washed in SMM (25% sucrose, 20 mM $MgCl_2$, 20 mM maleate, pH 6.5) and resuspended in SMM to 1/30 of the original volume. Protoplast transformation was done as described previously (16) except that protoplasts and DNA were incubated for 20 min at room temperature in 22.5% polyethylene glycol (18).

Southern hybridization. DNA on a 0.5% agarose gel was transferred to nitrocellulose filters as described by Southern (33). *Bam*HI fragment B and the 1.4-Md *Bam*HI/*Hin*dIII fragment of pWV05 were purified from digests of pGD4 and pGD6, respectively, after separation on 1% agarose gels. The fragments were nick translated with [α-^{32}P]dCTP (3,000 Ci/mmol; Radiochemical Centre) according to Rigby et al. (31). Hybridization of 0.5 μg of the fragments with the filters was performed overnight at 42°C in 25 ml of a solution containing 50% formamide, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 5× SSC (0.75 M NaCl plus 0.075 M sodium citrate), 1% sodium dodecyl sulfate, and 250 μg of denatured calf thymus DNA per ml. After hybridization, the filters were washed twice for 5 min at room temperature with 2× SSC, twice for 30 min at 65°C with 2× SSC containing 0.5% sodium dodecyl sulfate, and twice for 30 min at room temperature with 0.1× SSC. After drying, the filters were exposed to XAR-5 (Kodak) films.

CIE. *B. subtilis* and *S. lactis* were grown overnight in 500 ml of TY broth containing 5 μg of erythromycin per ml and in glucose-M17 broth with 1 μg of erythromycin per ml, respectively. Cells were harvested by centrifugation, washed with cold (4°C) 50 mM potassium phosphate (pH 6.8), and resuspended in 2 ml of the same buffer, containing 1 mM EDTA. The cells were disrupted by use of a Sonifier (MSE Ltd.) at the maximum power setting for four 30-s pulses. Triton X-100 was added to a final concentration of 4%, and after incubation for 30 min at room temperature, cell debris was removed by centrifugation for 15 min at 15,000 rpm in a Beckman SW41 rotor at 4°C. The cell-free extracts were concentrated by acetone precipitation. Protein concentration was determined by the method of Bradford (2). CIE of the cell-free extracts in the presence of antibodies raised against the purified proteins of the *S. cremoris* Wg2 proteolytic system was carried out as described previously (9). The gels were run at 2.5 V/cm for 3 h in the first dimension and at 1.5 V/cm for 16 to 18 h in the second dimension.

Growth and acid production in milk. Overnight cultures

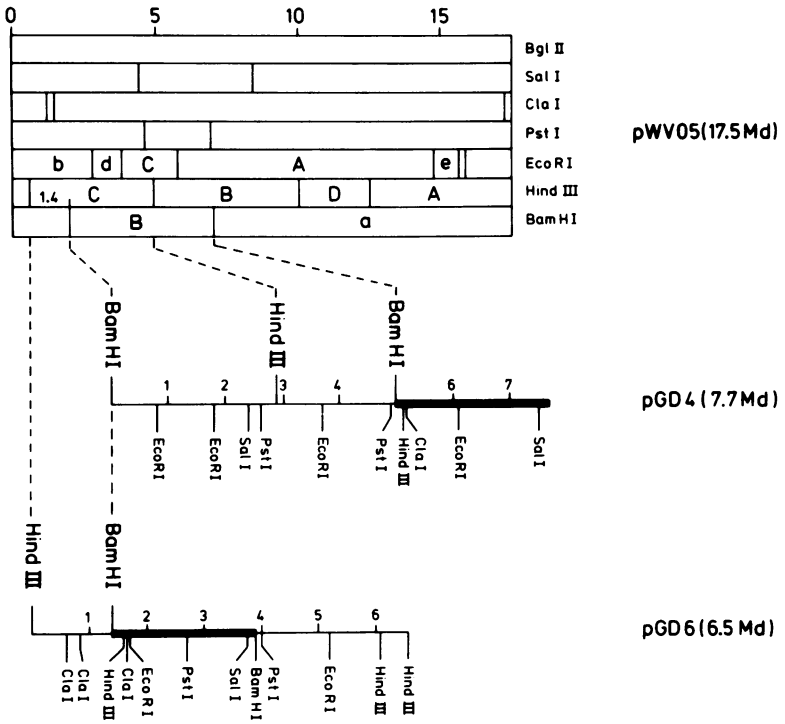

FIG. 1. Linear restriction enzyme maps of pWV05 and the recombinant plasmids pGD4 and pGD6. The three maps are calibrated in megadaltons. The unique *Bgl*II site of pWV05 was chosen to map at zero position. Those fragments cloned into pBR329 or pACYC184 are shown in capitals with the exception of *Hin*dIII fragment C, which was cloned in pGKV2. The vector DNA in pGD4 and pGD6 (pACYC184 and pBR329, respectively) is shown in bold lines. pGD4 and pGD6 are drawn as reproduced to show that together they comprise *Hin*dIII fragment C. For details, see text.

grown in glucose-M17 broth were washed in sterile distilled water and diluted 100-fold in skim milk (10%, wt/vol) containing 0.5% glucose. Samples were taken at 1-h intervals during incubation at 30°C. CFU were determined by plating on glucose-M17 agar, and acidity was determined by neutralization with 0.1 N NaOH.

RESULTS

Subcloning of plasmid pWV05. Gel electrophoresis of the total plasmid DNA of *S. cremoris* Wg2 and subsequent KI gradient centrifugation of the excised pWV05 bands yielded approximately 1.5 μg of pWV05 per liter of culture. A restriction enzyme map derived from simultaneous or sequential digestions of purified pWV05 with several restriction endonucleases is presented in Fig. 1. *E. coli* plasmids pBR329 (2.69 Md; 6) and pACYC184 (2.65 Md; 4) were used to clone parts of pWV05. *Bam*HI fragment B (5.0 Md) was cloned in pACYC184 (giving plasmid pGD4; Fig. 1), as were *Eco*RI fragments A (9.0 Md) and C (2.0 Md) (not shown). *Hin*dIII fragments A (5.2 Md), B (5.1 Md), and D (2.6 Md) were cloned into pBR329 (not shown). *Hin*dIII fragment C (4.3 Md) and *Eco*RI fragment B (4.6 Md) were never found among the recombinant vectors obtained, after either shotgun cloning or ligation of the specific fragments to the vector DNA. To clone the remaining part of pWV05, a *Bam*HI/*Hin*dIII double digest of total *S. cremoris* Wg2 plasmid DNA was ligated to *Bam*HI/*Hin*dIII-cut pBR329. One recombinant was isolated which contained the 1.4-Md *Bam*HI/*Hin*dIII fragment constituting the left part of *Hin*dIII fragment C. The right-hand *Bam*HI/*Hin*dIII fragment of *Bam*HI fragment B and a 0.5-Md *Hin*dIII fragment of unknown origin were also present in this scrambled plasmid (pGD6 in Fig. 1). These results, showing that breaking of the continuity of *Hin*dIII fragment C and *Eco*RI fragment B enabled the cloning of their subfragments in *E. coli*, suggest that either a particular property of the DNA sequence interfered with plasmid stability or expression interfered with the viability of *E. coli*.

Homology of pWV05 fragments with a proteinase plasmid of *S. cremoris* HP. On the basis of the pH and temperature optima of their proteinases, *S. cremoris* strains are divided into five different groups. According to this classification *S. cremoris* strains HP and Wg2 belong to the same group (11). Furthermore, it has been reported that *S. cremoris* HP carries a 9-Md proteinase plasmid (20). To investigate whether *Hin*dIII fragment C might specify proteinase activity, we examined its possible hybridization to the 9-Md proteinase plasmid of *S. cremoris* HP. To that purpose, total plasmid DNA of proteinase-proficient and proteinase-deficient strains of both *S. cremoris* HP and *S. cremoris* Wg2 was separated on a 0.5% agarose gel and transferred to nitrocellulose. The 5.0-Md *Bam*HI fragment from pGD4 and the 1.4-Md *Bam*HI/*Hin*dIII fragment from pGD6 were isolated from restriction enzyme digests. They were nick translated with [α-^{32}P]dCTP and used as probes in Southern hybridizations (33). The results are presented in Fig. 2. Both fragments hybridized only to plasmid pWV05 of *S. cremoris* Wg2 (Prt$^+$) and gave no signal with *S. cremoris* Wg2 (Prt$^-$), indicating that they were fragments of pWV05 and did not share homology with other plasmids of this strain. Furthermore, neither fragment hybridized with the plasmids of *S. cremoris* HP (Prt$^-$). They did, however, hybridize to the 9-Md proteinase plasmid present in *S. cremoris* HP (Prt$^+$). This observation, together with the fact that we were not

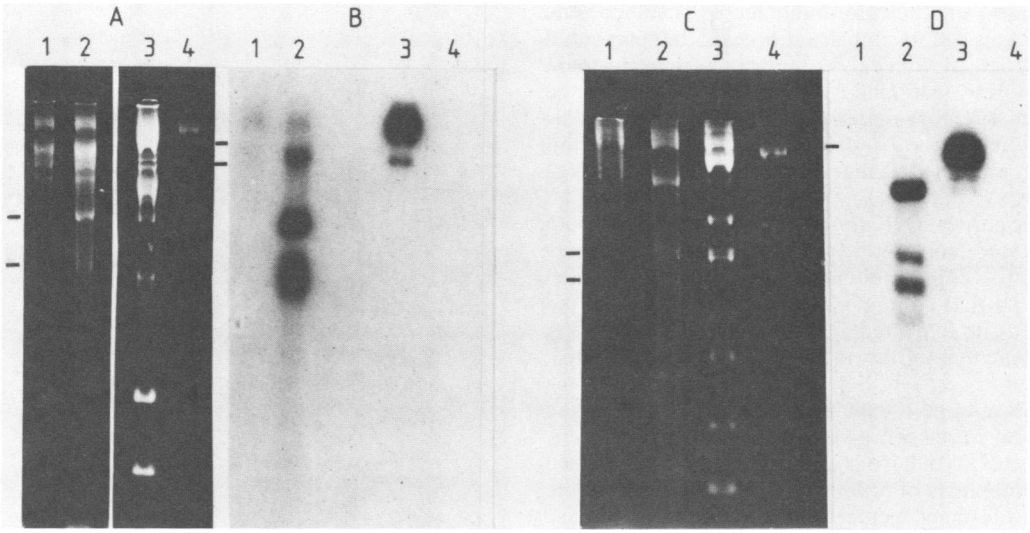

FIG. 2. Agarose gel electrophoresis and DNA-DNA hybridization of plasmids from *S. cremoris* strains. (A and C) Agarose gel (0.5%) electrophoresis of plasmid DNA from: lane 1, *S. cremoris* HP (Prt$^-$); lane 2, *S. cremoris* HP (Prt$^+$); lane 3, *S. cremoris* Wg2 (Prt$^+$); lane 4, *S. cremoris* Wg2 (Prt$^-$). (B and D) Autoradiogram of Southern blots of (A) and (C), respectively. The probe used in (B) was *Bam*HI fragment B of pWV05. The probe used in (D) was the 1.4-Md *Bam*HI-*Hin*dIII fragment of pWV05. The covalently closed and open circles of the proteinase plasmid in the *S. cremoris* HP and *S. cremoris* Wg2 isolates are marked at the left- and right-hand sides of the agarose gels, respectively.

able to clone *Hin*dIII fragment C in an uninterrupted way in *E. coli*, suggested a possible localization of the proteinase gene(s) on this fragment, assuming that expression of the gene(s) is lethal in *E. coli*.

Cloning in *B. subtilis*. Because the proteinases of lactic streptococci are cell wall-bound enzymes (10, 35) and therefore are subject to membrane transport, we tried to clone *Hin*dIII fragment C of pWV05 in the protein secretor and proteinase producer *B. subtilis*. Figure 3 shows a map of pGKV2 (3.1 Md), the cloning vector used for this purpose. pGKV2 is a derivative of the cryptic *S. cremoris* Wg2 plasmid pWV01 (16) and contains the erythromycin resist-

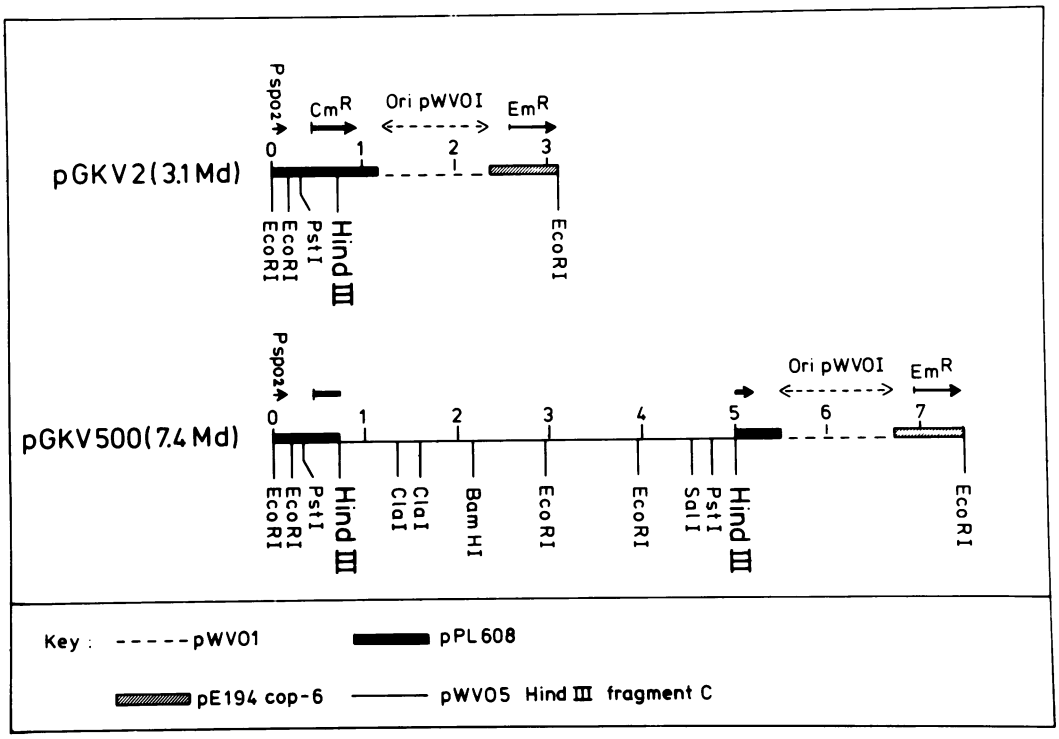

FIG. 3. Restriction enzyme maps of pGKV2 and pGKV500. The maps are linearized at the same *Eco*RI site to map at zero position. pGKV500 is pGKV2 with the 4.3-Md *Hin*dIII fragment of pWV05 inserted into its unique *Hin*dIII site.

ance gene of pE194 and the chloramphenicol resistance gene of pPL608. Details of its construction will be presented elsewhere (J. M. B. M. van der Vossen et al., manuscript in preparation). pGKV2 contains a unique *Hin*dIII site in the coding sequence of chloramphenicol acetyltransferase. This site was used to insert the *Hin*dIII fragment, electrophoretically purified from a *Hin*dIII digest of total *S. cremoris* Wg2 plasmid DNA.

After transformation of *B. subtilis* PSL1 protoplasts with the ligation mixture, erythromycin-resistant transformants were screened for chloramphenicol sensitivity. One such colony was found that contained a plasmid of the expected size and showed the expected *Hin*dIII digestion pattern. A restriction enzyme map of this plasmid, pGKV500 (7.4 Md), is shown in Fig. 3.

Transfer of plasmid pGKV500 to *S. lactis*. pWV01-derived vectors transform *B. subtilis* as well as *E. coli* and *S. lactis* (16). Therefore, pGKV500 isolated from *B. subtilis* was used to transform protoplasts of *S. lactis* MG1363, a plasmid-free strain, unable to ferment lactose and to produce proteinase because of the loss of a 33-Md lactose-proteinase plasmid (12). Rapid plasmid DNA extraction performed on five randomly picked transformants revealed that they all contained a plasmid with the same molecular weight as pGKV500 from *B. subtilis* (data not shown).

Characterization of protein specified by *Hin*dIII fragment C. From one of these *S. lactis* strains carrying pGKV500 and from *B. subtilis*(pGKV500) cell-free extracts were prepared and analyzed in CIE experiments. Extracts separated in the first dimension reacted with antibodies raised against the proteins of the purified proteolytic system of *S. cremoris* Wg2 (Prt^+) in the second dimension. CIE patterns are shown in Fig. 4 (*B. subtilis*) and 5 (*S. lactis*).

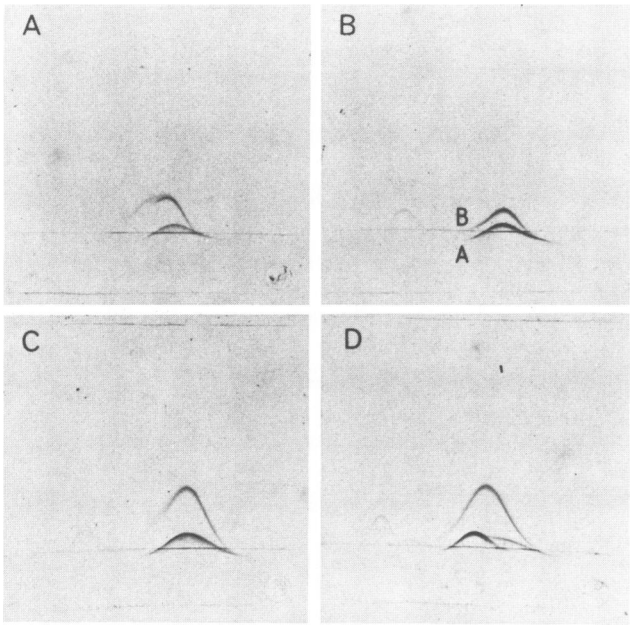

FIG. 4. CIE of a cell-free extract of *B. subtilis* PSL1(pGKV500) (A) and of the purified proteolytic system of *S. cremoris* Wg2 (B). Tandem CIE of cell-free extracts of *B. subtilis* PSL1(pGKV500) and of the purified proteolytic system of *S. cremoris* Wg2 applied in one well (C) and in the right and left wells, respectively (D). A 12-μl portion of the *B. subtilis* extract (42 mg of protein per ml) and 3 μl of the *S. cremoris* isolate (210 μg of protein per ml) were used. The second-dimension gel contained 30 μl of 28 mg of antibody per ml against the proteolytic system of *S. cremoris* Wg2.

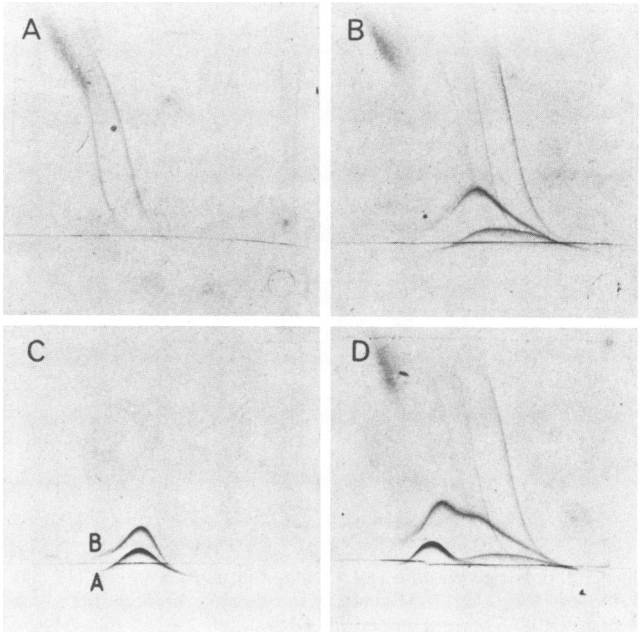

FIG. 5. CIE of a cell-free extract of *S. lactis* MG1363(pGKV2) (A), *S. lactis* MG1363(pGKV500) (B), and the purified proteolytic system of *S. cremoris* Wg2 (C). Tandem CIE of cell-free extract of *S. lactis* MG1363(pGKV500) (right well) and of the purified proteolytic system of *S. cremoris* Wg2 (left well) (D). A 15-μl portion of the *S. lactis* (pGKV2) extract (45 mg of protein per ml), 15 μl of the *S. lactis*(pGKV500) (53 mg of protein per ml), and 5 μl of the *S. cremoris* Wg2 isolate (210 μg of protein per ml) were used. The second-dimension gel contained 30 μl of 28 mg of antibody per ml against the *S. cremoris* Wg2 proteolytic system.

Figures 4B and 5C show the two major precipitation lines of the *S. cremoris* Wg2 proteolytic system, denoted A and B. Protein A has been shown to possess caseinolytic properties (14). The weak line at the left of A and B is an impurity not related to the proteolytic system (14). Figures 4A and 5B show that *B. subtilis*(pGKV500) and *S. lactis*(pGKV500) also produce two proteins which precipitate with the antibodies. Both proteins were absent in *S. lactis* (pGKV2) (Fig. 5A) and *B. subtilis*(pGKV2) (not shown). The precipitation lines running off the gel in the *S. lactis* extracts were present in both *S. lactis*(pGKV2) and *S. lactis*(pGKV500) and are, therefore, not insert specified (cf. Fig. 5A and B). The relationship between the insert-specified proteins and proteins A and B of *S. cremoris* Wg2 was examined in tandem CIE experiments.

With the *B. subtilis*(pGKV500) extract and the *S. cremoris* Wg2 isolate loaded in the same sample hole (Fig. 4C), only two peaks were visible, suggesting that the proteins in the upper and lower peaks of *B. subtilis*(pGKV500) are components A and B of the *S. cremoris* Wg2 proteolytic system, respectively. The observation that the surface under the two tandem peaks was roughly the sum of the individual peaks strengthens this view. Figures 4D and 5D show the results of a tandem CIE when the samples were applied on the same gel in adjacent holes. In Fig. 5D the precipitation line of *S. cremoris* Wg2 protein B completely fused with the upper peak in the *S. lactis*(pGKV500) extract, indicating that these two proteins are identical. In Fig. 4D the complete fusion of

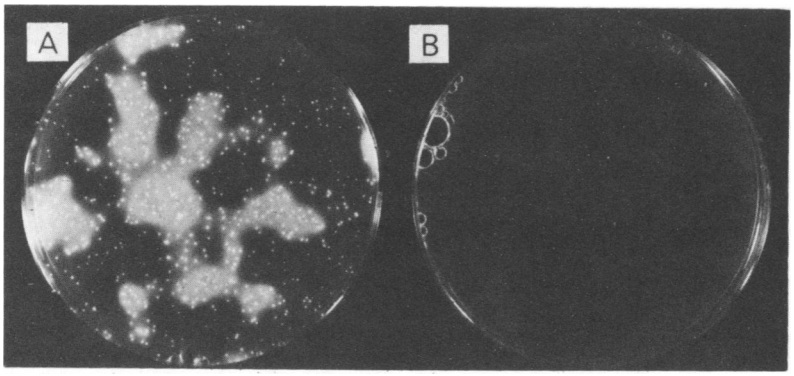

FIG. 6. *S. lactis* MG1363(pGKV500) (A) and *S. lactis* MG1363(pGKV2) (B) grown in transparent citrate-milk agar plates containing 0.5% glucose and 1 μg of erythromycin per ml. Incubation was for 48 h at 30°C.

S. cremoris Wg2 peak B with the *B. subtilis*(pGKV500) upper peak resulted in a higher and broader upper peak. Protein A from *S. cremoris* Wg2 shares common features with the protein in the lower precipitation line in both the *B. subtilis*(pGKV500) and the *S. lactis*(pGKV500) preparations: in both cases the peaks partially fused, the extract peak being lifted up. Apparently, protein A from *S. cremoris* Wg2 has more antigenic determinants than the protein in the lower peak in both cell-free extracts, causing it to precipitate under the extract peaks in a somewhat more diffuse way. From these experiments we conclude that *Hin*dIII fragment C from pWV05 carries the genes for proteins A and B of the *S. cremoris* Wg2 proteolytic system. The genetic information for protein B is complete, whereas the gene for protein A seems to be only partially present on this fragment.

Phenotype of *S. lactis*(pGKV500) in milk. Lactic streptococci are nutritionally fastidious and require an exogenous supply of many amino acids. The concentration of free amino acids and small peptides in milk limits growth and concomitant acid production. For optimal growth lactic streptococci are dependent on their proteinases, which hydrolyze milk protein (21). Media developed to differentiate between proteinase-positive and -negative variants rely on their difference in growth and acid production in milk. To investigate whether the *Hin*dIII fragment could restore good growth and acid production in a Prt^- host, growth on milk-based agar plates and acid production in milk of *S. lactis*(pGKV500) were examined. When suspended in transparent citrated milk agar (3), *S. lactis*(pGKV500) forms typical large Prt^+ colonies surrounded by a white precipitate of casein caused by rapid growth and acidification. *S. lactis*(pGKV2) only forms very small colonies characteristic of the Prt^- phenotype, even after prolonged incubation at 30°C (Fig. 6).

On GMAB medium *S. lactis*(pGKV500) gives large bright-yellow colonies with a yellow halo, whereas *S. lactis*(pGKV2) forms smaller colonies, white or faintly yellow, without a halo (not shown).

Growth and acid production in milk of *S. lactis*(pGKV500), *S. lactis*(pGKV2), and *S. cremoris* Wg2 were compared. *S. lactis* 712, the parental strain of the plasmid-free *S. lactis* MG1363 strain used throughout this study, carrying the 33-Md lactose-proteinase plasmid pLP712 (12), was also included in this comparison (Fig. 7). Because the first two strains are lactose deficient, the milk was supplemented with 0.5% glucose. Whereas *S. lactis*(pGKV2) stopped growing at a cell density of about 5×10^8 CFU/ml and produced only little acid, both growth and acid production in *S. lactis*(pGKV500) were enhanced to wild-type levels. These experiments indicate that *Hin*dIII fragment C of *S. cremoris* Wg2 plasmid pWV05 contains information for a functional proteinase activity.

DISCUSSION

The complexity of plasmid profiles in lactic streptococci makes it difficult to unambiguously assign certain host functions to specific plasmids (7, 8, 19, 30). Results of curing studies are often difficult to interpret, and confirmation of plasmid linkage of a particular property should be sought by other means such as cotransfer of plasmid and character (7)

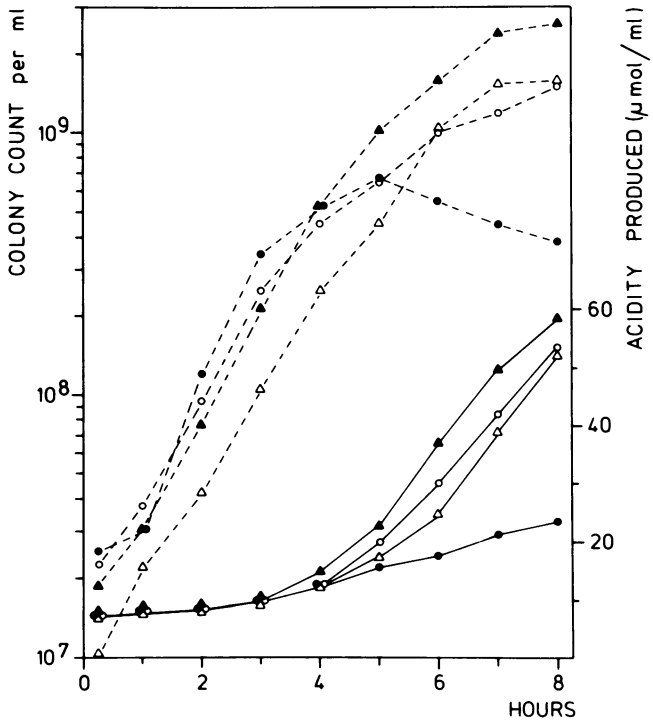

FIG. 7. Effect of plasmid pGKV500 on *S. lactis* growth and acid production in milk. Growth (– – –) and titratable acidity (——) are plotted against time. Data are shown for *S. cremoris* Wg2 (Prt^+) (△) and for *S. lactis* 712, carrying a complete plasmid complement (▲), and its derivatives *S. lactis* MG1363(pGKV500) (○) and *S. lactis* MG1363(pGKV2) (●).

or the construction of single-plasmid strains from a multiple-plasmid strain (12). Previous curing data strongly suggested the association of plasmid pWV05 of *S. cremoris* Wg2 with protein breakdown (29). The results presented here on the cloning of a 4.3-Md *Hin*dIII fragment of pWV05 and the association of this fragment with proteinase activity in *S. lactis* for the first time confirm the location of a suspected plasmid-encoded function through transfer of plasmid DNA, using genetic engineering techniques.

Several observations support the conclusion that the 4.3-Md *Hin*dIII fragment C of pWV05 carries the genetic information for proteinase production. First, *Hin*dIII fragment C showed homology with a plasmid suspected to encode proteinase activity in *S. cremoris* HP (20). *S. cremoris* strains have been classified in different groups on the basis of their proteolytic activities (11). According to this classification *S. cremoris* Wg2 and *S. cremoris* HP belong to the same group. It still remains to be elucidated, however, whether these proteinase activities are identical enzymes. Recently, Hugenholtz et al. (14) purified the proteolytic systems of various *S. cremoris* strains and characterized the proteins immunologically. They showed that at least one of the proteins, present in all strains, has caseinolytic activity. This protein, denoted A, and a second protein, B, are present in the proteolytic systems of *S. cremoris* Wg2 and *S. cremoris* HP.

Second, direct evidence that *Hin*dIII fragment C is associated with proteinase activity was obtained after successful cloning of the fragment in *B. subtilis* and *S. lactis*. The fragment specified two proteins in both organisms as revealed by CIE experiments with antibodies specific for the *S. cremoris* Wg2 proteolytic system. These proteins were identified as proteins A and B of the *S. cremoris* Wg2 proteolytic system. The gene for protein B is entirely present on the *Hin*dIII fragment (Fig. 5D), whereas the genetic information for protein A seems incomplete (Fig. 4D and 5D). Alternatively, the gene for protein A has been cloned entirely, but because of an undefined posttranslational modification step, the protein produced in *B. subtilis* and *S. lactis* differs from that produced by *S. cremoris*.

Third, conclusive evidence for proteinase linkage to the 4.3-Md *Hin*dIII fragment of pWV05 was obtained from growth experiments with *S. lactis* carrying the recombinant plasmid pGKV500. Under the conditions used, i.e., growth in milk supplemented with 0.5% glucose, the lactic streptococcus strains used depended critically on their proteolytic system for good growth. The data showed that the insert in pGKV500 restored the proteinase deficiency in *S. lactis* MG1363 and supported normal growth and acidification. This suggested that, if the gene for protein A was only partially present, it still specified a functional proteinase or that protein B was also a proteinase. Experiments to discriminate between these possibilities and to examine whether a chance fusion of the gene for protein A with the chloramphenicol acetyltransferase gene has occurred are in progress.

That the proteolytic activity of *S. cremoris* Wg2 is specified by *Hin*dIII fragment C offers an explanation for the failure to clone this fragment in *E. coli*. Expression of lactic acid streptococcal phospho-β-galactosidase has been demonstrated recently (M. J. Gasson and S. Maeda, Abstr. CEC Meet., Marseille, France, p. 34–35, 1984). It is conceivable that expression of the lactic streptococcal proteinase gene(s) is lethal in *E. coli*, considering the nature of the protein and the fact that it is transported through the membrane in lactic streptococci (10, 35). This was supported by the observation that pGKV500, having the replication functions of pWV01, which also function in *E. coli* (16), gave only very few erythromycin-resistant *E. coli* transformants, whereas pGKV2 transformed *E. coli* efficiently (unpublished data). In the few transformants obtained, pGKV500 has undergone extensive deletions and these are used now to further locate the genes for proteins A and B.

A useful practical expansion of this work for dairy culture genetics is the possibility of using *Hin*dIII fragment C or its subfragments as proteinase gene probes. In addition to hybridization to the *S. cremoris* HP proteinase plasmid, the fragments also hybridized to *S. cremoris* SK11 proteinase plasmid pSK111 (W. M. de Vos, personal communication). Moreover, the fragments hybridized to the *Bgl*II-D fragment known to carry the proteinase gene(s) of the *S. lactis* 712 proteinase-lactose plasmid pLP712, and they have been successfully used to identify proteinase plasmids in industrially important *S. cremoris* strains (W. D. Willis, personal communication).

The results presented here show the usefulness of the pWV01-derived vector family in indirect cloning in *S. lactis* and in the analysis of the genetics of lactic acid streptococci.

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